

PATENT
036835-46297

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Patent Application of :
Pascal Sebastian Bailon : Art Unit : 1647
Serial No. : 09/604,938 :
Filed : June 27, 2000 : Examiner : R. DeBerry
Title : ERYTHROPOIETIN CONJUGATES :
:

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.132 OF PASCAL SEBASTIAN BAILON

I, Pascal Bailon, a citizen and resident of the United States, hereby declare that:

I am the Pascal Bailon, the sole inventor named in the above-captioned patent application SN 09/604 938 filed June 28 2002 and familiar with the contents of this application (hereinafter "The Captioned Application") ;

In 1958, I received a B.Sc. in Chemistry from the University of Madras, Madras India;

In 1964, I received a M.S. in Chemistry from the University of Notre Dame, Notre Dame, Indiana;

From 1964-1966, I was a Biochemist at University Hospital, Cleveland, Ohio; and later at St. Luke's Hospital, Cleveland, Ohio;

From 1966-1969, I was a Biochemist at Georgetown University, Washington, D.C.;

From 1969-1970, I was a Biochemist at National Drug Company;

From 1970-1971, I was a Biochemist at Ciba-Geigy;

Since 1972, I am employed by Hoffmann-LaRoche Inc., Nutley, New Jersey, where my present position is Research Director for Biopharmaceuticals:

During my employment with Hoffmann-LaRoche Inc., Nutley, New Jersey, I have been involved with the expression, quantification, purification and biological testing of various recombinant proteins, which have included GCSF, EPO, interferons as well as the various conjugates of these proteins with polyethylene glycol (hereinafter referred to as PEG),.

The Captioned Application is directed to PEG conjugates of the protein EPO where the amino group of the EPO protein is conjugated to the PEG via an amide bond and a linking group which contains 2 to 3 carbon atoms separating the amide bond from the PEG substituent .

This Declaration is submitted to demonstrate that

- a. The EPO-PEG amide conjugates of this invention linkage by a 2 to 3 carbon atom separation between the amide bond and the PEG moiety have new and unexpected properties as compared to PEG-EPO conjugates containing other types of linkages; and

b. The EPO-PEG amide conjugates of this invention where the amide bond is separated by 3 carbon atom linkage from the PEG moiety, produces new and unexpected properties over the corresponding conjugate linked by a 2 carbon atom separation between the PEG moiety and the amide bond:

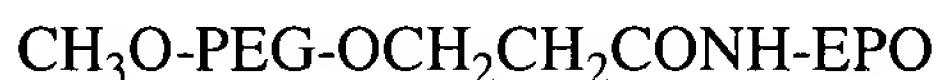
The new and unexpected results are demonstrated through their biological activity in vivo as determined by the ability of these EPO conjugates to increase production of reticulocytes in the blood as measured by the normocythaemic mouse assay.

Reticulocytes are young red blood cells showing a basophilic reticulum under vital staining and an increase in reticulocytes translates into an increase in the production of red blood cells.

The normocythaemic mice assay measures ability of a test compound to increase the production of reticulocytes in the blood and thereby increase the production of red blood cells.

In order to demonstrate that the EPO conjugates which are linked in accordance with the invention of The Captioned Application produces new and unexpected results over EPO conjugates utilizing other type of linkages, the following EPO conjugates of this invention were prepared under my direction and control.

Compound 1. Methoxy-PEG-SPA (Succinimidyl propionic acid)

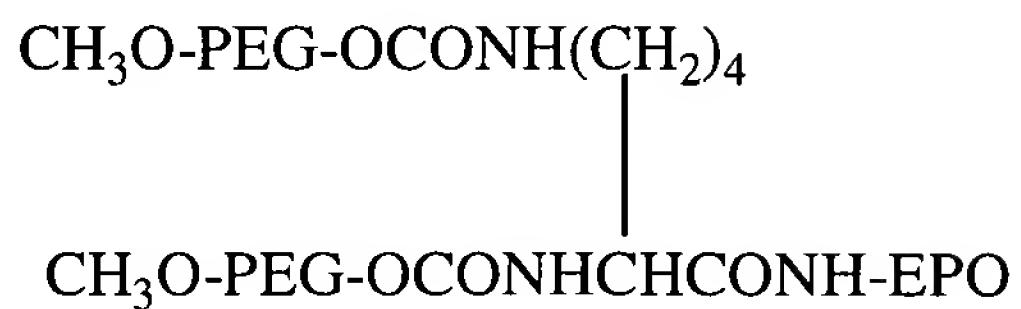


Compound 2. Methoxy-PEG-SBA (Succinimidyl butyric acid)

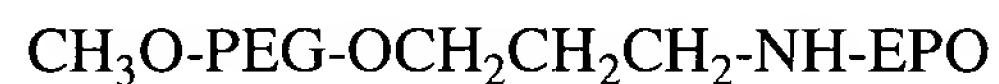
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and compared with the following conjugates:

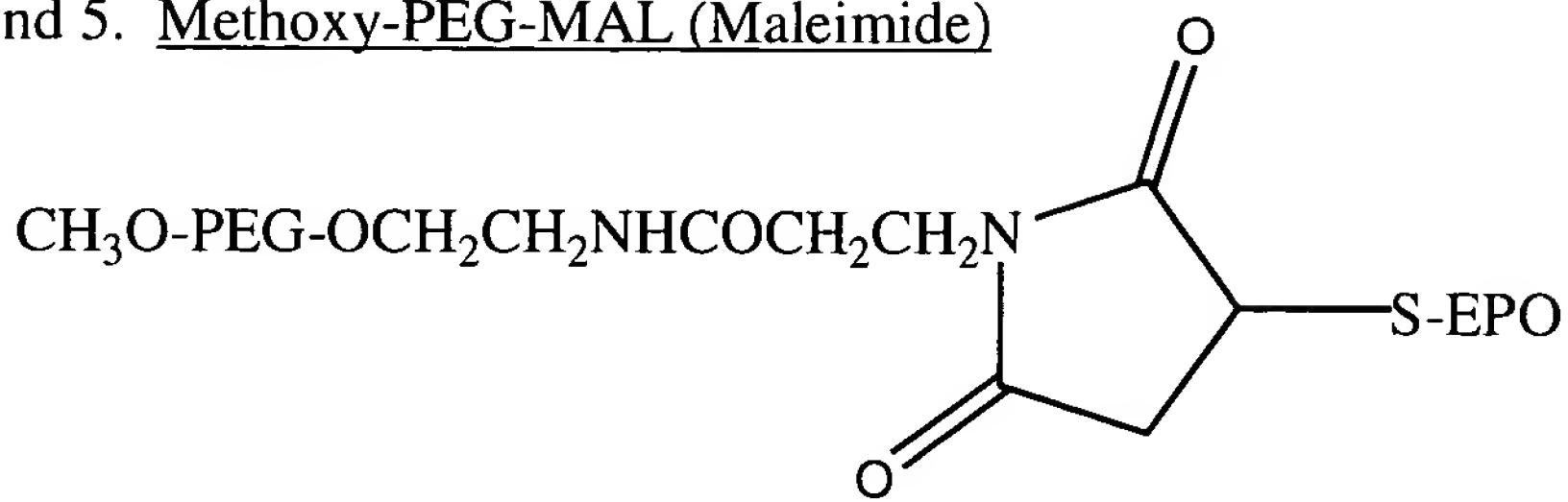
Compound 3. PEG₂-NHS (Branched-methoxy-PEG succinimide)



Compound 4. Methoxy-PEG-ALD (Aldehyde)



Compound 5. Methoxy-PEG-MAL (Maleimide)

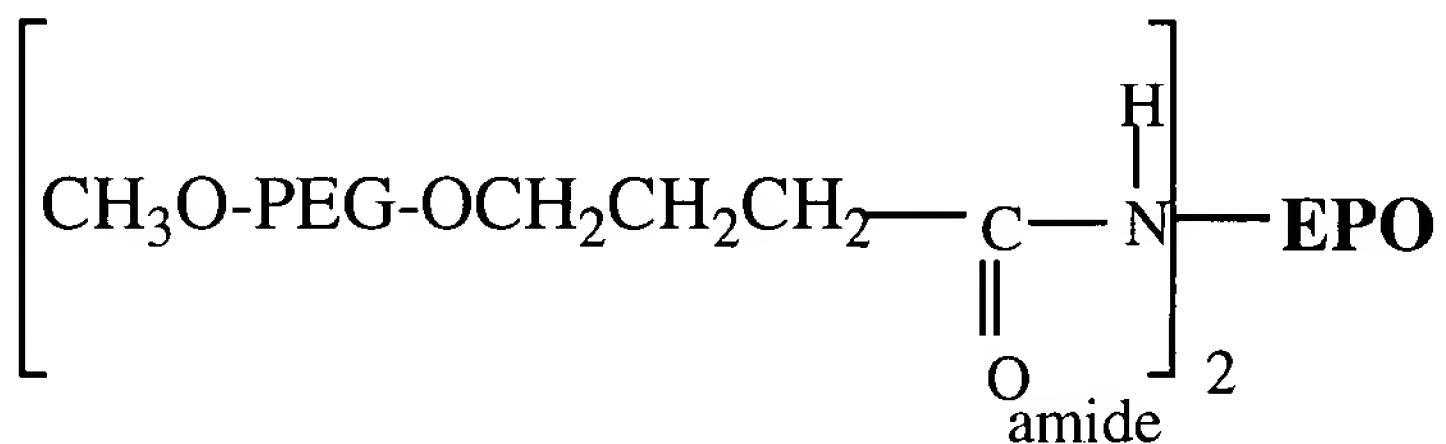


which compounds were also prepared under my direction and control.

Compound 3 uses the divalent linker disclosed in US Patent No 6,025,324, Bailon et al.; Compound 5 is an EPO conjugate disclosed in PCT application WO 01/02017, Burg et al January 21 2001

Compounds 1 and 2 were also compared with the divalent conjugate which is part of the invention in the captioned Application.

Compound 6.



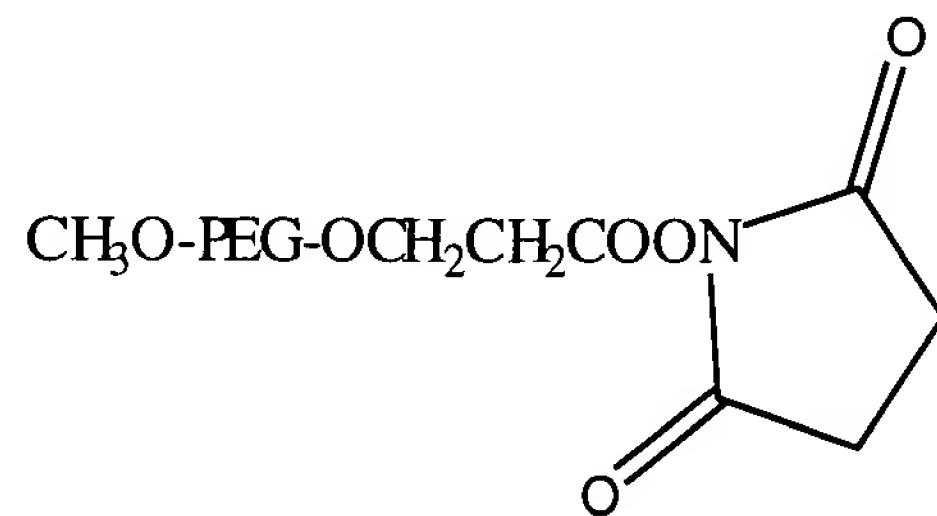
All of the above compounds were subjected to normocytthaemic mouse assay test and the results were reported to and reviewed by me.

Experiment

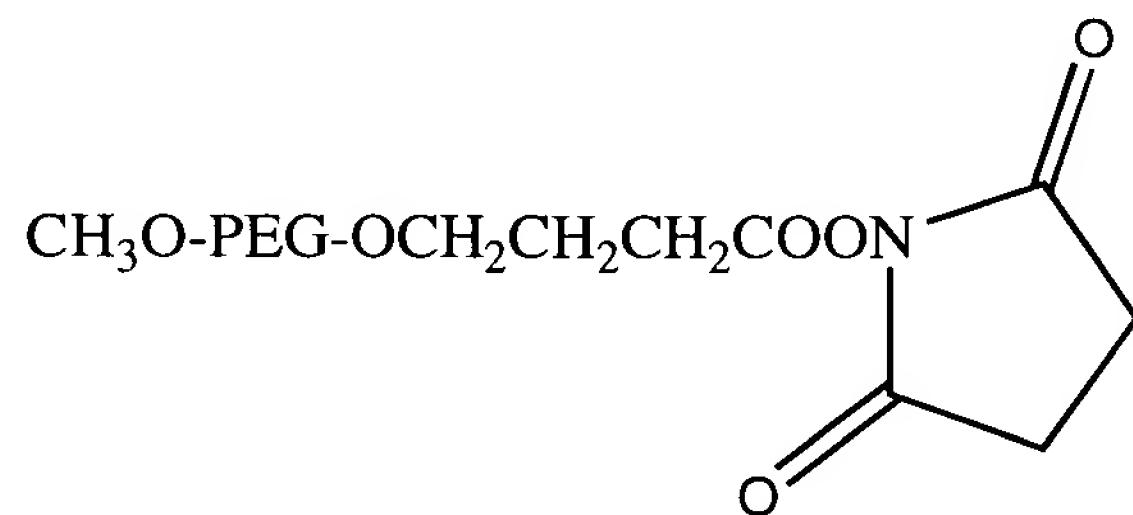
The EPO utilized all of the conjugates and the compound was prepared in accordance with Example 1 of the Compound Application.

The reagents utilized for producing the conjugates tested were the following:

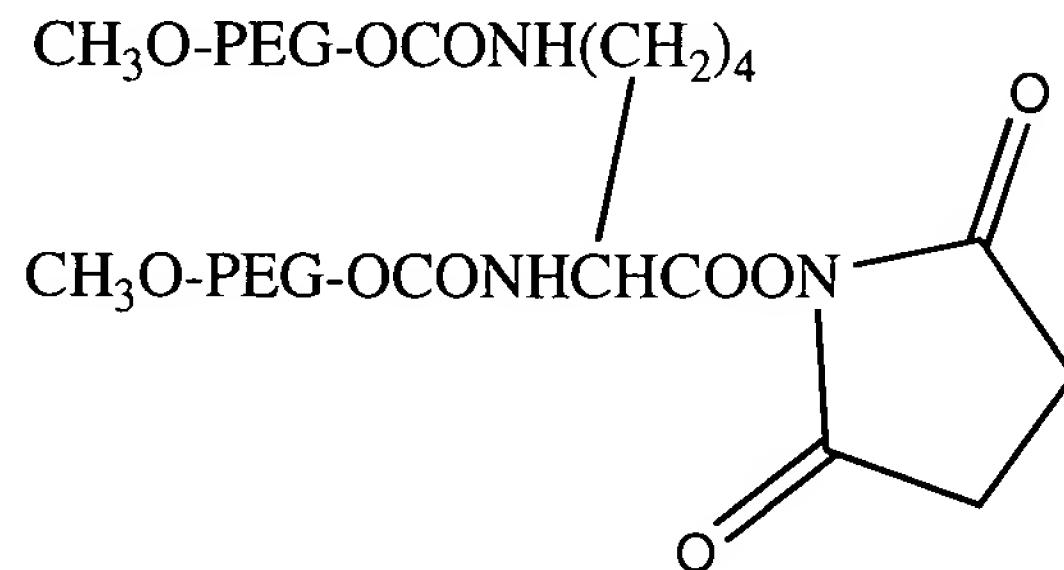
Reagent 1. Methoxy-PEG-SPA(Succinimidyl propionic acid) where PEG has a molecular weight ("MW") of 30 kDa



Reagent 2. Methoxy-PEG-SBA(Succinimidyl butyric acid) PEG MW = 30 kDa



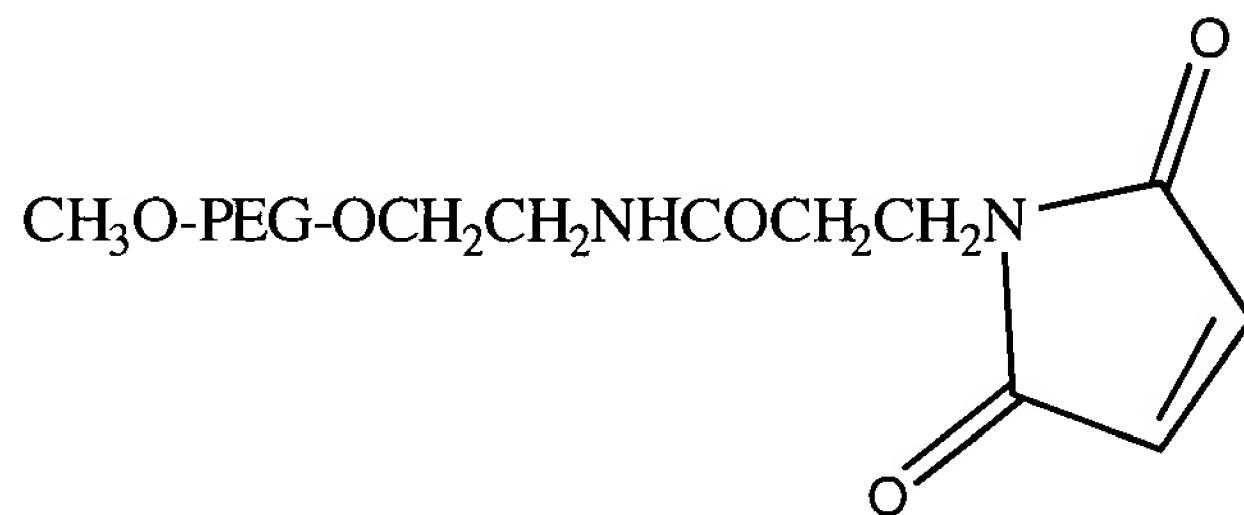
Reagent 3. PEG₂-NHS (branched-methoxy-PEG succinimide) where each PEG has a MW = 20 kDa



Reagent 4. Methoxy-PEG-ALD (Aldehyde) PEG MW = 20 kDa



Reagent 5. Methoxy-PEG-MAL (Maleimide) PEG MW = 30 kDa



Procedures For Preparation of Conjugates

1. **30 kDa m-PEG-SPA-EPO (Compound 1).** Twenty-five milligrams of EPO was dialyzed into 100 mM sodium borate buffer pH 8.0. This compound was prepared by the procedure of Example 2 of the Captioned Application utilizing Reagent 1. To the dialyzed EPO solution enough borate buffer was added to make up the volume of 5 mL. The final protein concentration was 5 mg/mL. Forty-one milligrams (1.36 micromols) of PEG-SPA was added to the dialyzed 25 mg EPO (1.36 micromols). Final EPO: PEG-

SPA molar ratio was 1:1. The reaction mixture was shaken for 4 h at 4°C. The reaction was terminated by adjusting the reaction mixture pH to 4.5 with acetic acid.

The PEG-EPO was separated from the reaction byproducts and remaining unmodified EPO by cation exchange chromatography. The reaction mixture was diluted 100-fold with distilled water and applied to 2.5x4cm S-Sepharose column, previously equilibrated with 10 mM sodium acetate pH 4.5 at a flow-rate of 10 mL/min. Unadsorbed materials were washed away with the equilibration buffer. PEG-EPO was eluted with 0.2M NaCl in the equilibration buffer and the strongly bound unmodified EPO was eluted with 0.5M NaCl in the same buffer. The pegylated EPO of Compound 1 was concentrated to ~1 mg/mL and diafiltered into 10 mM potassium phosphate buffer pH 7.5 and stored frozen at - 20°C.

2. m-PEG-SBA-EPO (Compound 2). Reaction and purification conditions were the same as in PEG-SPA-EPO except the Reagent 2 was utilized to produce Compound 2.

3. m-PEG2-EPO (Compound 3). Twenty-five milligrams of EPO (1.36 μ mol) was dialyzed into 100 mM potassium phosphate buffer pH 7.5 and the final volume was made up to 5 mL with the same buffer to a final protein concentration of 5 mg/ mL. One hundred and eight milligrams of 40 kDa PEG2-NHS [Reagent 3] (2.72 μ mol) reagent was added to the EPO solution. The final EPO: PEG2-NHS mole ratio was 1:2. The reaction was performed at 4°C for 4h. The reaction was terminated as described and PEG2-EPO (Compound 3) was purified as before.

4. m-PEG-ALD-EPO (Compound 4). Ten milligrams of EPO (0.54micro moles) was dialyzed into 100 mM potassium phosphate pH 5.0 and made up to 5 mL with the same buffer, to a protein concentration of 2 mg/mL. Two hundred and seventy-one milligrams (13.5 μ mol) of 20 kDa PEG-ALD was [Reagent 4] reacted with the EPO solution. The EPO:PEG-ALD molar ratio

was 1:25. To the reaction mixture 250 μ L of a 1 M sodium cyanoborohydride solution in water was added and mixed for 6h at room temperature. PEG-ALD-EPO [Compound 4] was purified as before.

5. m-PEG-MAL-EPO (Compound 5). EPO was reacted with N-succinimidyl-S-acetylthioacetate (SATA) to introduce thiol groups. 1.388 micromols (0.32 mg) of SATA was dissolved in 20 μ L DMSO and immediately added to 5 mg EPOsf solution in 0.5 mL potassium phosphate containing 50 mM NaCl, pH 7.5. SATA: EPOsf molar ratio was 5:1. The reaction mixture was incubated at 25°C for 30 min. PEG-MAL (33.3 mg) was dissolved in 0.5 mL 10 mM potassium phosphate buffer, containing 50 mM NaCl, 4 mM EDTA, pH 7.5. It was added directly to the EPO/SATA reaction mixture. Thirty μ L of a fresh 1M hydroxylamine was added to initiate the coupling reaction. The reaction mixture was stirred for 90 min at room temperature. Two μ L of a 1M stock solution of Cysteine was added and mixed for 30 min to terminate the coupling reaction. Five μ L of a 1M N-methyl maleimide was mixed with the mixture for 30 min to block any unreacted thiol groups on the pegylated EPO. The final reaction mixture was dialyzed against 10mM potassium phosphate pH 7.5. PEG-MAL-EPO (Compound 6) was purified on a S-Sepharose column as described earlier.

Preparation of predominantly mono-PEG-EPO Pegylation Reaction

To 100 mg (5.48 mol) of EPO in 100 mM potassium phosphate buffer pH 7.5, 329 mg (10.96 mol) of 30 kDa PEG-SBA was added. Enough 100 mM potassium phosphate buffer pH 7.5 was added to make the reaction mixture volume to 20 mL. The final protein concentration was 5 mg/mL and the protein to PEG reagent ration was 1:2. The reaction mixture was mixed for 2h at room temperature (20-22 C). After 2h, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid, and stored frozen at -20 C until ready for purification.

Purification

The reaction mixture from the previous step was diluted with 10mM sodium acetate, pH 4.5 and applied to 300mL SP-Sepharose FF (sulfopropyl cation exchange resin) packed into a 4.2 x 19 cm column, which was previously equilibrated with the same buffer. Column effluents were monitored at 280 nm with a Gilson UV monitor and recorded with a Kipp and Zonen recorder. The column was washed with 300 mL, or 1 column volume of equilibration buffer to remove excess reagents, reaction byproducts and oligomeric PEG-EPO. This was followed by washing with 2 bed volumes of 100 mM NaCl to remove di-PEG-EPO. Mono-PEG-EPO was then eluted with 200 mM NaCl. During elution of the mono-PEG-EPO, the first 50 mL of the protein peak was discarded and the mono-PEG-EPO was collected as a 150 mL fraction. Unmodified EPOsf remaining on the column was eluted with 750 mM NaCl. All elution buffers were made in the equilibration buffer.

Preparation of Endogenous Gene Activated EPO

EPO_{ega} produced by endogenous gene activation technology, which can activate, downregulate or amplify, any gene of interest. In this process, EPO was produced by the upregulation of quiescent EPO genes by insertion of a new regulatory element into human cells to change gene expression pattern.

HELaS3 cell derived EPO_{ega} was produced by a serum-free fermentation process and purified using multiple column chromatography steps which enabled the separation and collection of the basic isoforms 5 to 8 of EPO_{ega}. Due to differences in glycosylation of these basic isoforms, especially lower sialylation, the specific activity was lower and corresponded to only 80,000IU/mg. The EPO was conjugated with a 30

kDa PEG Reagent 2 to produce compound 2 and with 40 kDa Reagent 4 to produce Compound 4. In addition this EPO_{ega} was used to prepare

Compound 7. Methoxy-PEG-HZ (Hydrazide)



starting from

Reagent 6. Methoxy-PEG-HZ (Hydrazide) PEG MW = 5 kDa



by the following procedure

7. **m-PEG-HZ-EPO (Compound 7).** Oxidation Step: To 10 mg EPO_{ega} 5.04 mL of a 100 mM sodium acetate buffer pH 5.5 was added. It was mixed with 10 mg/mL sodium meta-periodate in the acetate buffer. The total volume was 7.66 mL and the final periodate concentration was 10mM. The mixture was allowed to sit on ice for 30 min. Excess periodate was quenched by the addition of 3.3 mL of 80 mM sodium sulfite, which was allowed to stand for 5 min. In compound 7, PEG is conjugated to a sugar in EPO.

Desalting: The oxidized EPO solution was applied to a 1.4x24 cm Sephadex G-25 (fine) column which was equilibrated with 100 mM sodium acetate pH 4.3 at a flowrate of 1 mL/min. The peak containing the oxidized EPO was collected and protein concentration was determined.

Pegylation: To 20 mL desalted oxidized EPO (10 mg) 250 mg (100 molar excess) PEG-HZ was added and mixed for 4h at 4°C and the solution was stored at -20°C until ready for purification.

Purification: Reaction mixture was diluted 5-fold and pH adjusted to 7.5 with dilute NaOH. It was then applied to a 1x6cm DEAE Sepharose FF column equilibrated with PBS buffer pH 7.3. The unadsorbed materials were washed away with the equilibration buffer containing 80 mM NaCl. The PEG-HZ-EPO (Compound 7) was eluted with 150 mM NaCl in the equilibration buffer.

Testing Procedure

The biological evaluation, used the normocytthaemic mouse bioassay set forth in Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2)) and the method in the monography of erythropoietin of Ph. Eur. BRP was used. The samples of compounds 1 through 7 were diluted with BSA-PBS. Normal healthy mice, 7 to 15 weeks old, were administered s.c. 0.2 ml of the EPO-fraction containing un-pegylated EPO or tri-, di- or mono-pegylated EPO from Example 2 or 3. Over a period of 6 days, blood was drawn by puncture of the tail vein and diluted such that 1 μ l of blood was present in 1 ml of an 0.15 μ mol acridine orange staining solution. The staining time was 3 to 10 minutes. The reticulocyte counts were carried out microfluorometrically in a flow cytometer by analysis of the red fluorescence histogram. The reticulocyte counts were given in terms of absolute figures (per 30,000 blood cells analyzed). For the data presented, each group consisted of 5 mice per day, and the mice were bled only once. As controls, unmodified EPO and a buffer solution were administered in the same manner as set forth above. The results of Compounds 1 through 7 and the controls with an unmodified EPO and the buffer solutions are given in Table I.

The conjugates prepared from endogenous gene activated EPO was evaluated by the above procedure. The results are given in Table 2.

RESULTS

TABLE 1
Production of Reticulocytes induced by Various PEG-EPO Preparation

<u>Blood Collection</u>	<u>Vehicle</u>	<u>EPO</u>	<u>Compound No.</u>					
			<u>2</u> <u>30 SBA</u>	<u>1</u> <u>30K SPA</u>	<u>3</u> <u>40K PEG2</u>	<u>4</u> <u>20K ALD</u>	<u>5</u> <u>30K MAL</u>	<u>6</u> <u>Di 30K SBA</u>
72h	250	950	1350	1393	1111	1200	1133	994
96h	240	1210	1920	1406	1021	1300	1720	926
120h	220	1000	2033	1100	936	900	1467	944
144h	215	590	2133	660	743	950	1233	694

10ng/mouse
mean values (n=5)

TABLE 2
Production of Reticulocytes induced by Endogenous EPO-PEG Conjugates

<u>Blood Collection</u>	<u>Compound No.</u>		
	<u>2</u> <u>30 K SBA</u>	<u>4</u> <u>20 K ALD-PEG (ega)*</u>	<u>7</u> <u>5 K HZ</u>
	<u>PEG (ega)</u>		
72h	1227	1200	1000
96h	1417	1300	900
120h	935	900	850
144h	591	750	700

10 ng/mouse
mean values (n=5)

* determined in a separate assay

Discussion of Results

As seen from Table 1, all of the conjugates set forth increased reticulocytes production as compared to EPO, 72 hours after administration. However, the greatest 72 hours increase in reticulocytes production occurred with respect to the conjugates of

Compounds 1 and 2 of this invention, which when compared to the next active compound, compound 5, produced a substantial increase in reticulocytes production.

As seen from Table 1, 96 hours after administration, specific conjugates of this invention, Compounds 1 and 2, along with the conjugate of Compound 5 substantially increased the production of reticulocytes as compared to the control, EPO and as compared to the other conjugates.

As can be seen from the results of Table 1 the conjugates 1, 2, and 5 substantially increased the production of reticulocytes 120 hours after administration. In this 120 hour period, conjugates 1 and 2 of this invention had twice the activity as to producing reticulocytes as compared to the control and had far greater activity than all of the other conjugates with the exception of Conjugate 5. In addition, the conjugates 1 and 2 of this invention and conjugate 5 maintained their biological activity over a period of 120 hours as compared to the control whereas the other conjugates did not maintain their biological activity.

As seen from the results of Table 1 after 144 hours (6 days), Conjugate 2 of this invention maintained its biological activity whereas the other conjugates with the exception of Conjugate 5 did not maintain a substantial increase in biological activity as compared to the EPO control, and the biological activity of Conjugate 2 being more than 40% greater than the activity of the Compound 5 and three times more than the activity of Conjugate 1 at this time period.

As seen from the results in Table 1, the buturic acid conjugate of Compound 2 has substantially three times the activity of the propionic acid conjugate of Conjugate 1 and 40% more activity than Conjugate 5 which is more active than the other conjugates.

The results in Figure 2 are just presented for completion. There is no means for measuring the effectiveness of these results since no controls were used and there would be differences in activity between the EPO prepared recombinantly used in Table 1 and the EPO used in Table 2 which was produced by endogenous gene activation. Since there would be a difference in glycosylation, the endogenous gene activated EPO had a much lower specific activity than the corresponding recombinant produced EPO of Example 1.

Conclusions

As seen from the results in Table 1 the activity of the EPO-PEG amide conjugates depends upon the linkage and bond between the PEG moiety and the protein.

As seen from the results in Table 1 the EPO-PEG amide conjugates of this invention having a single amide bond linked by a two to three carbon atom separation between the amide bond and the PEG moiety have new and unexpected properties as compared to PEG EPO conjugates, other than Conjugate 5, containing other type of linkages, since these compounds are active for over 120 hours after administration to produce reticulocytes in the blood.

That as seen from the results in Table 1 the EPO-PEG amide conjugate of this invention where the amide bond is separated by a three carbon atom linkage from the

PEG moiety produces new and unexpected properties over the corresponding conjugate of this invention linked by a two carbon atom separation between the PEG moiety and the amide bond, since the three carbon atom linkage produces a conjugate which is far more active for 144 hours (6 days) than any of the other conjugates tested.

That I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Pascal Bailon

Pascal Bailon

Dated: 5/20/2002